Age- and gender-dependent myocardial transcription patterns of cytokines and extracellular matrix remodelling enzymes in cats with non-cardiac diseases

Fonfara, Sonja ; Hetzel, Udo ; Hahn, Shelley ; Kipar, Anja

Abstract: Background:Age, genderand systemic diseases all influence cardiac function and remodelling.In cats, age and genderassociated myocardial remodelling and the effect of systemic diseases on the myocardium have so far not been studied. The aim of the study was therefore to investigate whetherrelevant cytokines and extracellular matrix (ECM) remodellingenzymesare expressed in the myocardium of cats with non-cardiac diseasesand whether transcription levels are influenced by age and gender. Methods:The study was performed on myocardial samples from 26 cats aged between 2 and 19 years that had died with non-cardiac diseases. Seventeen cats were female (2 entire), nine were male (1 entire). Of these, nine cats were diagnosed with diseases unlikely to affect the myocardium (control cats). The remaining 17 cats suffered from diseases with likely systemic effects.All hearts were assessed for any pathological changes, and the myocardium was analysed for interleukin (IL)-1, -2, -4, -6, -18, tumour necrosis factor (TNF)- , interferon(IFN)- , transforming growth factor (TGF)- , matrix metalloproteinase (MMP)-2, -3, -13, tissue inhibitor of MMP (TIMP)-1, -2 and -3 transcription using quantitative RT-PCR assays. Results:Despite the absence of any histological evidence of myocardial damage,inflammation and fibrosis, the myocardium of all cats was found to constitutively transcribe cytokines and ECM remodelling enzymes, with generally higher mRNA concentrations in atria than in ventricles.Young and male cats exhibited higher transcription levels throughout the myocardium in comparison to older and female cats. Furthermore, age-associated transcription pattern differed between male and female cats. Conclusion:The constitutive transcription of ECM remodellingenzymes suggests continuous myocardial remodelling throughout the entire life of a cat. The myocardium of young and malecats appears to be in a pro-inflammatory state, whereas in older and female cats the myocardium exhibits a reduced inflammatory reaction to systemic disease.Age-associated cardiac remodelling seems to be influenced bynon-hormonal factors inmale and female cats.

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Methods: The study was performed on myocardial samples from 26 cats aged between 2 and 19 years that had died with non-cardiac diseases. Seventeen cats were female (2 entire), nine were male (1 entire). Of these, nine cats were diagnosed with diseases unlikely to affect the myocardium (control cats). The remaining 17 cats suffered from diseases with likely systemic effects. All hearts were assessed for any pathological changes, and the myocardium was analysed for interleukin (IL)-1, -2, -4, -6, -18, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, transforming growth factor (TGF)-β, matrix metalloproteinase (MMP)-2, -3, -13, tissue inhibitor of MMP (TIMP)-1, -2 and -3 transcription using quantitative RT-PCR assays.
Results: Despite the absence of any histological evidence of myocardial damage, inflammation and fibrosis, the myocardium of all cats was found to constitutively transcribe cytokines and ECM remodelling enzymes, with generally higher mRNA concentrations in atria than in ventricles. Young and male cats exhibited higher transcription levels throughout the myocardium in comparison to older and female cats. Furthermore, age-associated transcription pattern differed between male and female cats.
Conclusion: The constitutive transcription of ECM remodelling enzymes suggests continuous myocardial remodelling throughout the entire life of a cat. The myocardium of young and male cats appears to be in a pro-inflammatory state, whereas in older and female cats the myocardium exhibits a reduced inflammatory reaction to systemic disease. Age-

associated cardiac remodelling seems to be influenced by non-hormonal factors in male and female cats.

Point by Point Reply

We would like to thank both reviewers for the time and effort they dedicated to our manuscript and their useful comments, which helped to improve the quality of our manuscript. As recommended by reviewer 2, we have reviewed our analysis with one of our statisticians, who did agree with reviewer 2. We have removed the age groups,normalisedour results, incorporated gender into the analysis and recalculated our statistics. As recommended by both reviewers, we have completely rewritten the manuscript(except for the description of laboratory methods in material and methods as these did not change), removed the tables and changed the figures.We have therefore not marked the parts, which we did change, as the complete manuscript would be marked. We hope that we have addressed all issues and concerns of the reviewers to their satisfaction.

Reviewers' comments:

Reviewer #1: Fonfara and colleagues present an interesting analysis of cytokines and extracellular matrix in cats of various ages. The study appears to be well designed, and the authors are well published in this area. I cannot comment as a non-veterinarian on the selection of various cats and the assertion about which cats are likely to have cardiovascular disease.

Cats with cardiac disease were not included in the study. The cats of the study were seen as clinical patients and had an assessment by a veterinarian and in most cases a diagnosis prior to death. However, the clinical assessment of the animalswill have been limited in some cases due to the decline of further investigations by the owner.This was one of the reasons that a complete necropsy was performed to confirm and/or obtain a diagnosis. The heart was investigated by an experienced pathologist. We were therefore able to exclude cats with cardiac diseases, which would have influenced study results.

I think for the physician dealing with human patients that the study may have relevance but the discussion needs to be reworked. The mix of animal and human studies is difficult to follow. Perhaps the discussion could be divided into one section comparing this work to that of other animal studies and a separate (labelled) section for those who want to read implications for human research. It does appear that the information is in the current manuscript but it remains confusing after several readings.

We hope we managed to improve this, the manuscript was rewritten; however, occasionally we still use human/small animal model research studies as references. We use these, when no information/studiesforcats/dogs areavailable.

Reviewer #2: The authors investigated the mRNA transcription of selected cytokines and MMP/TIMP in cats of different ages and sought to identify expression patterns that might reflect the impact of age and systemic diseases on myocardial remodeling. From a multitude of data, they infer that ageing leads to less inflammatory response to systemic disease. The paper is well written and there are no major technical concerns. On the other hand, for those routinely involved in "human" biomedical research, several aspects appear rather unusual.

1. The animals were pet cats that were euthanized for a variety of reasons, which introduces a lot of variability and potential confounding factors compared to studies using animals bred for research purposes. In consequence, the "systemic diseases" are extremely heterogenous, with obvious impact on the validity and meaningfulness of the data.

We do agree with the reviewer. We used a normal cat population with several different diseases, which will have had an impact on results and a larger population of a homogenous group (only one systemic disease) would have been preferable. However, as we used pet animals, the selection of cats is limited and relies on owners willing to donate their pets for research. The advantage of this population of cats is that it reflects a normal cat population,in contrast to a purpose bred animal population, which might produce more homogenous results, but with limited relevance for clinical practice.Interestingly, despite ourheterogeneous group, we could detect significant differences between groups and identify the influence of age on myocardial marker transcription.
One of our aims is to homogenise groups to investigate the influence of specific diseases on cardiac remodelling in the cat. However, this was not the aim of the current study.

If so many different disease states may have an impact on myocardial behavior, what about, for example, diet and exercise? Both are known to significantly influence myocardial behavior, and nothing is known about it for the individual cat.

We do agree with the reviewer, diet and exercise will vary between cats. However, possibly not as much as in human medicine,as most cats are fed a commercial available diets and thesevary much less than the diet of individual humans. Furthermore, cats are generally not very activeand the effect of exercise might not be as marked as in humans, who exercise consciously for their health. But we agree there will be differences. We donot know about the diets and exercise levels of the cats included in our study, as we did not ask the owners for this information. We have added this to the limitations.
This will be an interesting aspect for further studies, as far as we are aware no one has looked into these factors in cat or dog pets.

2. Grouping for age is generally not a good idea. Data from all cats should be entered into a statistical model and the impact of age should then be tested as a one covariable by logistic regression etc.

The statistical analysis was changed, the age groups were removed.

3. I assume that much of the information given in Table 4 overlaps with that shown in the Figures and is therefore redundant. Such detailed tables may offer maximum transparency, but are extremely difficult to read.

All tables were removed, results are now in the text and infigures.

4. Similarly, the figures, while commendably detailed with their multitude of p-values and symbols, are too complicated for the "ordinary" reader. Another, more to-the-point i.e. simpler way to visualize the data so that they readily support the conclusions should be found.

All figures were changed.

5. In relation to the amount of data provided and their significance, both introduction and discussion section are too long.

The manuscript was rewritten, both parts are shorter.

Highlights

- Myocardial cytokine, MMP and TIMP transcription varies with age and gender.
- Age-dependent myocardial transcription differs in neutered male and female cats.
- The myocardium of young and male cats is in a pro-inflammatory state.
- Hearts of old and female cats show a reduced inflammatory reaction.
- Cytokine and ECM remodelling enzyme transcription differs in atria and ventricles.

Age-and gender-dependent myocardial transcription patterns of cytokines and extracellular matrix remodelling enzymesin cats with non-cardiac diseases

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Abstract

*Background:*Age, genderand systemic diseases all influence cardiac function and remodelling.In cats, age and genderassociated myocardial remodelling and the effect of systemic diseases on the myocardium have so far not been studied. The aim of the study was therefore to investigate whetherrelevant cytokines and extracellular matrix (ECM) remodellingenzymesare expressed in the myocardium of cats with non-cardiac diseasesand whether transcription levels are influenced by age and gender.

*Methods:*The study was performed on myocardial samples from 26 cats aged between 2 and 19 years that had died with non-cardiac diseases. Seventeen cats were female (2 entire), nine were male (1 entire). Of these, nine cats were diagnosed with diseases unlikely to affect the myocardium (control cats). The remaining 17 cats suffered from diseases with likely systemic effects.All hearts were assessed for any pathological changes, and the myocardium was analysed for interleukin (IL)-1, -2, -4, -6, -18, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, transforming growth factor (TGF)-β, matrix metalloproteinase (MMP)-2, -3, -13, tissue inhibitor of MMP (TIMP)-1, -2 and -3 transcription using quantitative RT-PCR assays.

*Results:*Despite the absence of any histological evidence of myocardial damage,inflammation and fibrosis, the myocardium of all cats was found to constitutively transcribe cytokines and ECM remodelling enzymes, with generally higher mRNA concentrations in atria than in ventricles.Young and male cats exhibited higher transcription levels throughout the myocardium in comparison to older and female cats. Furthermore, age-associated transcription pattern differed between male and female cats.

*Conclusion:*The constitutive transcription of ECM remodellingenzymes suggests continuous myocardial remodelling throughout the entire life of a cat. The myocardium of young and malecats appears to be in a pro-inflammatory state, whereas in older and female cats the

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- Myocardial cytokine, MMP and TIMP transcription varies with age and gender.
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- Cytokine and ECM remodelling enzyme transcription differs in atria and ventricles.

Keywords (up to 6): myocardium; cytokine transcription; cardiac remodelling; age; extracellular matrix; MMP and TIMP expression.

1. Introduction

In the human heart, ageing is characterised by progressive ventricular fibrosis and cardiomyocyte density, resulting in increased ventricular stiffness and diastolic dysfunction(Kitzman and Edwards, 1990). In the ageing human population heart failure with preserved ejection fraction is a frequent presentation. Gender differences are well known, with female species exhibiting favourable cardiac pathology (Chua et al., 2011; Pavon et al., 2012).Furthermore, systemic diseases such as diabetes mellitus, renal diseases, hypertension, and obesity, are known comorbidities and contribute to impaired cardiac function (Ather et al., 2012). Similar presentations are seen in cats and dogs. Age associated increased ventricular stiffness is observed in pet animals (Santilli and Bussadori, 1998; Schober and Fuentes, 2001; Saunders, 2012), retrospective studies suggest a male predisposition forhypertrophic cardiomyopathy in cats (Atkins et al., 1992; Abbott, 2010; Payne et al., 2010)and older animals develop systemic diseases that are known heart failure comorbidities in human patients (Metzger and Rebar, 2012; Saunders, 2012).

Increased ventricular stiffness is caused by imbalanced extracellular matrix (ECM) remodelling and ventricular fibrosis (Spinale, 2007). The composition of the ECM is regulated by enzymes, the matrix metalloproteinases (MMP), which degrade the ECM components, and their inhibitors, tissue inhibitors of MMP (TIMP-1 to -4), which indirectly lead to ECM deposition(Vanhoutte and Heymans, 2010). Matrix metalloproteinase and TIMP imbalance and ECM degradation and deposition are associated withcardiac diseases and dysfunction (Spinale, 2007), but onlylittle is known about their role in age related cardiac changes. Mouse models ofageingexhibit varying MMP, TIMP and transforming growth factor (TGF)-β mRNA and protein production (Brooks and Conrad, 2000; Lindsey et al., 2005; Tian et al., 2007; Kandalam et al., 2010; Wang et al., 2010; Givvimani et al., 2013; Uchinaka et al., 2014).

Cytokines play an important role in the activation of MMPs and TIMPs. They contribute to the regulation of inflammatory responses and are elevated in cardiac and systemic diseases (Hedayat et al., 2010; Fonfara et al., 2012;Fonfara et al., 2013a). In particular interleukin (IL)-6 and tumour necrosis factor (TNF)-α are linked to ageing processes and were found to be elevated in patients with loss of muscle mass and cachexia (Morley and Baumgartner, 2004; Walston et al., 2009). However, their main role during ageing is not yetclear(Maggio et al., 2006).

No studies exist investigating the influence of age or gender on the myocardium in cats. Gaining knowledge of the myocardial ageing and remodelling processes in cats is of particular interest, not only because the systemic diseases that often affect ageing cats are similar to known human comorbidities (i.e. diabetes mellitus, renal diseases, systemic hypertension), but also because cats are commonly neutered, which allows the investigation of non-hormonal gender associated cardiac remodelling.

The objective of the present study was to investigate the constitutive, i.e. non-cardiac disease associated transcription of a range of cytokines and ECM remodelling enzymes known to be relevant for cardiac remodelling in other species, in different cardiac regions and in catsof different gender and age. A proportion of cats had systemic diseases that potentially affected cardiac function or resulted in a systemic inflammatory response. The transcription patterns of these cats were therefore compared to the baseline expression of cats with diseases unlikely to affect the heart.

2. Material and methods

2.1.Animals and tissues

Twenty six cats were included in the study. Cats were patients that had been presented at the Universities of Helsinki and Bristol without clinical evidence of cardiac disease and

had been euthanised upon owner's request due to poor prognosis, impaired quality of life or financial constraints. Informed consent was obtained from owners prior to inclusion into the study and cats were assigned arbitrary numbers as identifiers. Institutional ethical approval was obtained.

From each cat, thesignalement including breed, sex, age, weight and body condition score (BCS) was recorded.The heart was removed within one hour after death and grossly examined which confirmed the absence of any apparent cardiac disease. Myocardial samples from the interventricular septum, right atrium and ventricle, and left atrium and ventricle were collected for RNA extraction and stored in RNA stabilising solution (RNAlater;Ambion, Life Technologies, Paisley, UK) at -20°C until analysed. Hearts were subsequently fixed in 10% formalin and samples from the same sites as those for RNA extraction were prepared and routinely paraffin wax embedded for histological examination. Sections (3-5 µm thick) were prepared and stained with haematoxylin-eosin (HE).

Twenty three cats subsequently underwent full necropsy to identify any relevant disease conditions. Samples were collected from all major organs as well as any gross abnormalities, fixed in 10% formalin and routinely paraffin wax embedded for histological examinationof HE stained sections.For the remaining three cats (all from group "control cats") owners did not give consent for a full necropsy and only dissection and histological examination of the heart was permitted.

Based on the clinical and pathological findings, cats with diseases unlikely to have an effect on cardiac function and/or unlikely to have systemic effects, such as the systemic release of cytokines, that could have induced cytokine, MMP and/or TIMP production in the myocardium, were considered as control cats (group "control cats"); all remaining cats were allocated to group "systemic diseases", implying that they suffered from conditions with

potential systemic effects, which might have influenced myocardial transcription processes (Fonfara et al., 2013a, b).

2.2.RNA extraction

After removal from RNAlater, total RNA was extracted from the myocardial samples using a commercially available kit (Qiagen RNeasy Plus Universal mini Kit, Manchester, UK) according to the manufacturer's protocol. An initial step was added in which the tissue was placed in liquid nitrogen; the frozen tissue was then transferred into 900 µL lysis reagent and ground thoroughly with a tissue pestle grinder. An on-column DNA digestion step was included. Final elution of the total RNA was performed using 30 µL of RNase-free water and repeated to maximize the amount of eluted RNA. The total RNA concentration of each sample was determined with a spectrophotometer.

2.3.Real time PCR

cDNA was synthesised from 300 ng total RNA using the I Skript cDNA Synthesis Kit (Biorad, Hertfordshire, UK) according to the manufacturer's protocol and stored at -80°C until use in the quantitative PCR. For the feline housekeeping gene GAPDH, as well as feline IL-1, -6, -18, TNF-α and TGF-β1,primers of previously published sequences were used (Kipar et al., 2001; Van Nguyen et al., 2006; Taglinger et al., 2008). Primers for feline IL-2, IL-4, IFN-γ, MMP-2, -3, 13, TIMP-1, -2 and -3 were designed using Primer Express software (Applied Biosystems, Life Technologies), and BLAST searches performed to confirm gene specificity. Primer sequences are shown in Table 1. Primers were synthesised by Eurogentec (Hamphsire, UK). Primers were validated using a standard curve of eight serial dilutions and primer efficiencies were between 94% and 99%. PCR was performed according to standard protocols.Aliquots (1 µL) of cDNA were amplified in duplicates by PCR in 20 µL reaction

volumes on a real time Stratagene 3500(Applied Biosystems, Life Technologies) using Maxima SYBRGreen qPCR Master Mix (Fermentas, Cheshire, UK). Each assay well had a 20 µL reaction volume consisting of 12.5 µL SYBR Green PCR mastermix, each of 400 nM forward and reverse primers, and 1 µL of sample cDNA (templates) or water (negative controls). The amplification was performed according to standard protocols, with 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The PCR was followed by a dissociation programmewith 1 min at 95°C, succeeded by 41 cycles during which the temperature was increased by 1°C at each cycle, starting at 55°C and ending at 95°C. All PCR reactions exhibited one well-defined melting curve peak. Relative expression levels were normalised to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1
Primer sequences used for quantitative PCR.

Primer		Sequence (5'-3')
IL-2	Forward	CTGCTTCAAGCTCTACAAAGGAAAC
	Reverse	CTCCATTCAAAAGCAACCGTAAA
IL-4	Forward	TCGTCCACGGCCAGAACT
	Reverse	TTTCTCGCTGTGAGGATGTTCA
IFN-γ	Forward	ATGATGACCAGCGCATTCAA
	Reverse	TTTACTGGAGCTGGTATTTAACAACTTATC
MMP-2	Forward	TGGAGAGGCGGACATCATG
	Reverse	CCCGTCCTTGCCATCAAA
MMP-3	Forward	GTTCTGGGCCATGAGAGGAA
	Reverse	GGAAAACCCAGGGTGTGGAT
MMP-13	Forward	GACCCTCGACGCCATCAC
	Reverse	GCAGGCGCCAGAAGAATCT
TIMP-1	Forward	GATACTTGCACAGGTCCCAGAAC
	Reverse	TCCGTCCCGCAGGTTTC
TIMP-2	Forward	CTCCGGATGAATGTCTCTGGAT
	Reverse	GCAGAAGAACTTGGCCTGATG
TIMP-3	Forward	CAACAAATACCAGTACCTGCTGACA
	Reverse	GGTCCCACCTCTCCACAAAGT

IL: interleukin, IFN: interferon, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of matrix metalloproteinase.

2.4.Statistical analysis

For the statistical analysis of the PCR results, SPSS was used. Basic descriptive statistics were calculated for the parameters recorded and analysed. Age and weight were normally distributed. Other variables were log transformed to improve normality and the model assumptions necessary for parametric analysis.

No difference in transcriptions waspresent between the right and left atrium (data not shown), which were subsequently combined as “atrial samples”. The same was true for the three ventricular localisations (right and left ventricle and interventricular septum; data not shown) and these were combined as“ventricular samples”. These combined sample groups were subsequently used for the comparison of localisations.

Constitutive transcription was assessed in group “control cats”. Subsequently, to assess the effect of non-cardiac diseases with potential systemic effects, all group “systemic diseases” animals were compared with group “control cats”. Cardiac regions,groups and genderwere compared using 1-way ANOVA andunpaired t-tests as appropriate. Linear regression analysis was used to explore the relationship between the markers investigated and age and body weight. Results are displayed as mean and standard deviation. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Study population

Seventeen cats were female (2 entire), nine were male (1 entire). The age of the entire population ranged from 2 to 19 years with a mean of 9 years ± 6. No significant difference for age, weight or BCS was present between male and female cats (data not shown). Nine

cats (group “control cats”) had been euthanised due to behavioural abnormalities or because they exhibited local disease processes that the owners decided not to treat due to financial constraints (Table 2).This entire group subsequently served as control for the group “systemic diseases” cats with diseases that were considered to have potential systemic effects including, potentially, the induction of cytokine and ECM enzyme production in the myocardium (Table 2).No significant difference between groups was observed for age, weight and BCS (data not shown).

Table 2
Breed, sex, age, weight, body condition score (BCS) and main diagnosis of cats included in group “control cats”(cats with diseases unlikely to have systemic effects or influence cardiac function) andgroup “systemic diseases”(cats with diseases likely to have systemic effects or an influence on cardiac function).

Group	Breed	Sex	Age (years)	Weight (kg)	BCS (/5)	Diagnosis
Control cats	DSH	FN	2	4.3	3	Oesophageal stricture
	DSH	FN	2	4.5	3	Nasal polyp
	DSH	FN	3	5	3	Discus prolaps, no improvement post-surgery
	DSH	FN	9	5.8	4	Behaviour abnormalities
	DLH	FN	10	4	3	Behaviour abnormalities
	DSH	FN	10	3	2	Behaviour abnormalities
	DSH	FN	14	1.7	2	Nasal osteosarcoma
	DSH	M	14	4.6	3	Age-related non-specific changes
	DSH	FN	19	3.3	2	Gastrointestinal stromal tumour
Systemic diseases	DSH	F	2	3.5	3	Non-suppurative meningoencephalitis, chronic interstitial pancreatitis, interstitial nephritis, mild pericholangitis
	BSH	MN	3	n.r.	3	Lymphocytic cholangiohepatitis
	DSH	FN	4.5	3	2	Pulmonary adenocarcinoma
	DSH	MN	6	6.1	4	Astrocytoma
	Devon Rex	MN	7	n.r.	n.r.	Lymphoplasmacytichepatitis
	DSH	MN	8	6	4	Pancreatic islet amyloidosis, one endstage kidney
	Siamese	FN	8	n.r.	2	Mast cell tumour
	DSH	MN	9	4	2	Intestinallymphoma(with involvement of liver and lung)

	Oriental	FN	10	2.7	3	Mammary adenocarcinoma with lung metastases
	DSH	MN	10	4	2	Multicentric lymphoma
	DSH	FN	12	3.9	2	Diabetes mellitus-associated pancreatic and hepatic changes
	DSH	MN	13	6	3	Pyogranulomatous nephritis (FIP excluded)
	DSH	MN	13	4.4	3	Necrotising neutrophilic colitis with purulent lymphadenitis
	DSH	F	14	3	2	Marked chronic cholangiohepatitis, mild chronic interstitial pancreatitis and chronic enteritis
	DSH	FN	15	2.9	2	Adenocarcinoma lung, metastases in kidney, brain, pancreas
	DSH	FN	18	3.8	3	Chronic cystitis
	DSH	FN	19	2.7	3	End stage kidney, eosinophilic syndrome, chronic pancreatitis

BCS: body condition score, BSH: British short-hair cat, DLH: domestic long-hair cat, DSH: domestic short-hair cat, F: female, FN: female neutered, kg: kilogram, M: male, MN: male neutered, n.r.: not recorded.

3.2. Cytokines, MMPs and TIMPs are constitutively transcribed in the feline myocardium, with higher transcription levels in atria.

All examined cytokines, MMPs and TIMPs were found to be transcribed, with some variation in frequency and some degree of intra- and interindividualquantitative variation. IL-1, IL-2, IL-4, IL-18, IFN-γ, TGF-β, MMP-2, MMP-3, MMP-13, TIMP-2 and TIMP-3 mRNA was detected in all samples, i.e. both in atria and ventricles of all cats regardless of age, sex and disease, whereas IL-6, TNF-α, and TIMP-1 were expressed in 94%, 89%, and 81%, respectively.Among the cytokines, IL-18 and TGF-β exhibitedthe overall highest transcription levels, followed by IFN-γ, IL-2, IL-4, IL-1, IL-6, and TNF-α (Figure 1A). Among the ECM remodelling enzymes, TIMP-2 mRNA levels were highest, followed by TIMP-3, MMP-2, -3, -13 and TIMP-1 (Figure 1B). An assessment of individual cats (and diseases) did not identify any cases with overall higher cytokine, MMP or TIMP transcription levels; also, there was no evidence of disease associated expression patterns.

A comparison between atria and ventriclesshowed that transcription levels of all markers were generally significantly higher in the atria when the entire study population ($p<0.001$, Figure 1A, B) or the cats with systemic, non-cardiac diseases (group "systemic diseases") were examined. In the control cats (group "control cats"), this applied to most markers.

Fig. 1A

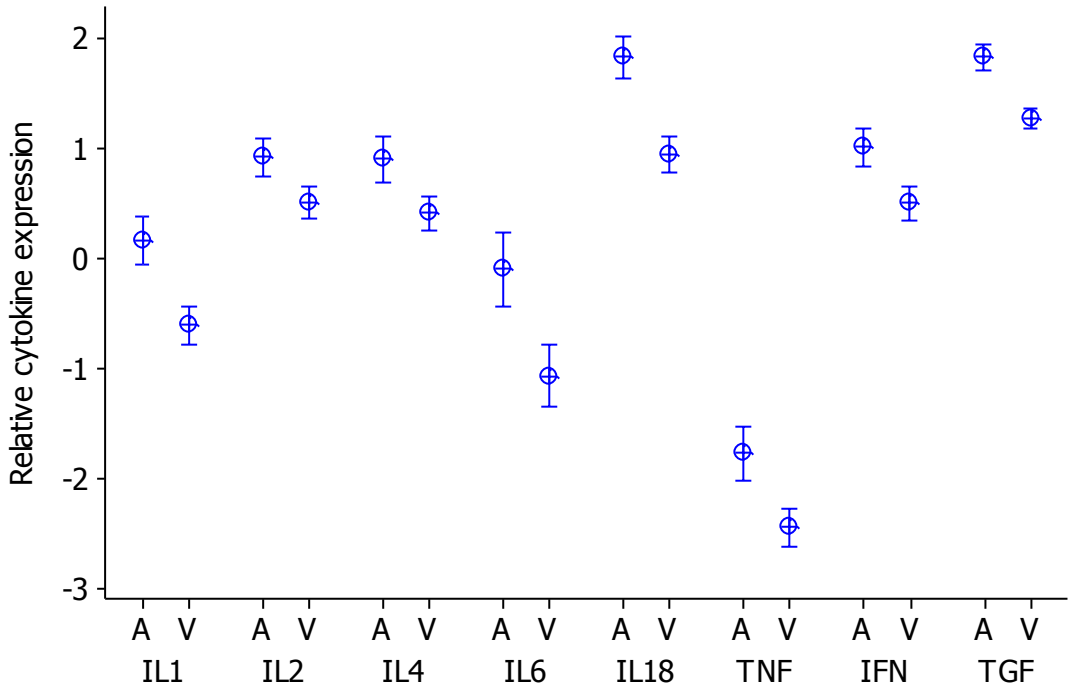

Fig. 1B

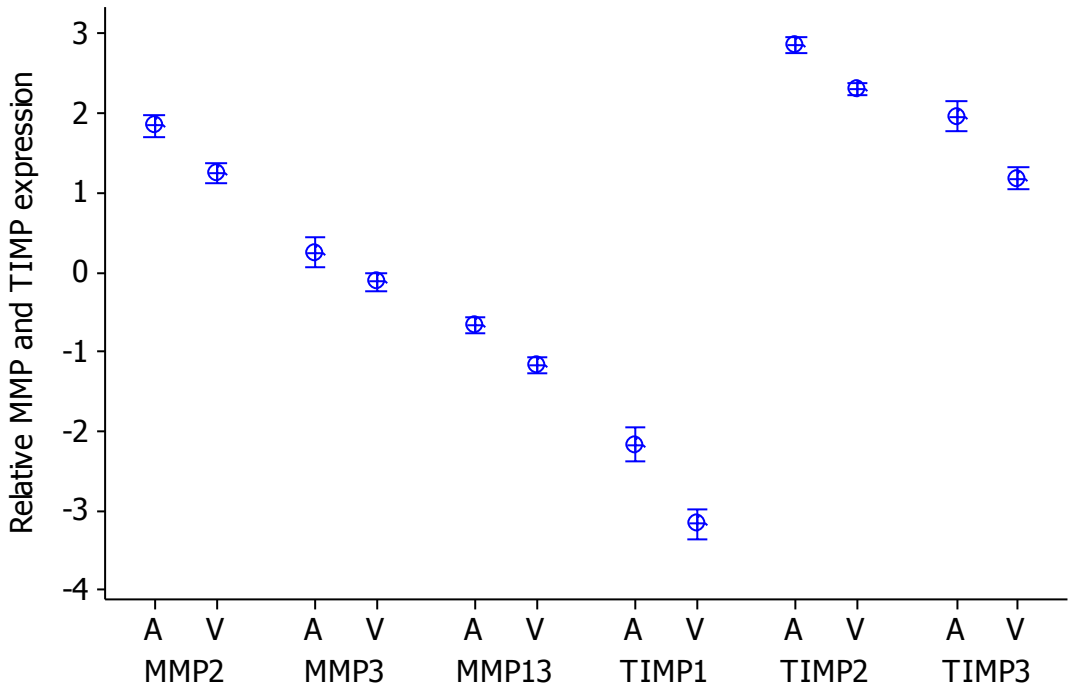

Fig. 1A, B. Relative mRNA expression of (**A**) IL-1, IL-2, IL-4, IL-6, TNF-α, IFN-γ, TGF-ß, and (**B**) MMP-2, MMP-3, MMP-13, TIMP-1, TIMP-2 and TIMP-3 comparing atria (A) ventricular (V) samples. Transcription levels of all markers were significantly higher in atria than ventricular samples ($p<0.001$). Error bars, bars represent 95% confidence interval for mean.

3.3. The myocardium exhibits age- and gender-associated quantitative differences in the expression of cytokines, MMPs and TIMPs

Age was negatively correlated with myocardial transcription for IL-1 ($p=0.008$), IL-2 ($p<0.001$), IL-4 ($p<0.001$), IL-6 ($p=0.013$), IFN-γ ($p=0.034$), MMP-3 ($p=0.004$). A positive correlation was present with TGF-β ($p=0.019$). This applied for atrial and ventricular samples for IL-4 ($p=0.01$ and $p=0.003$, respectively), the correlation for the other markers was only apparent in the ventricles (IL-1 $p=0.019$, IL-2 $p<0.001$, IL-6 $p=0.018$, IFN-γ $p=0.025$, TGF-β $p=0.001$, MMP-3: $p=0.008$; Figure 2 A-D).

Fig. 2A

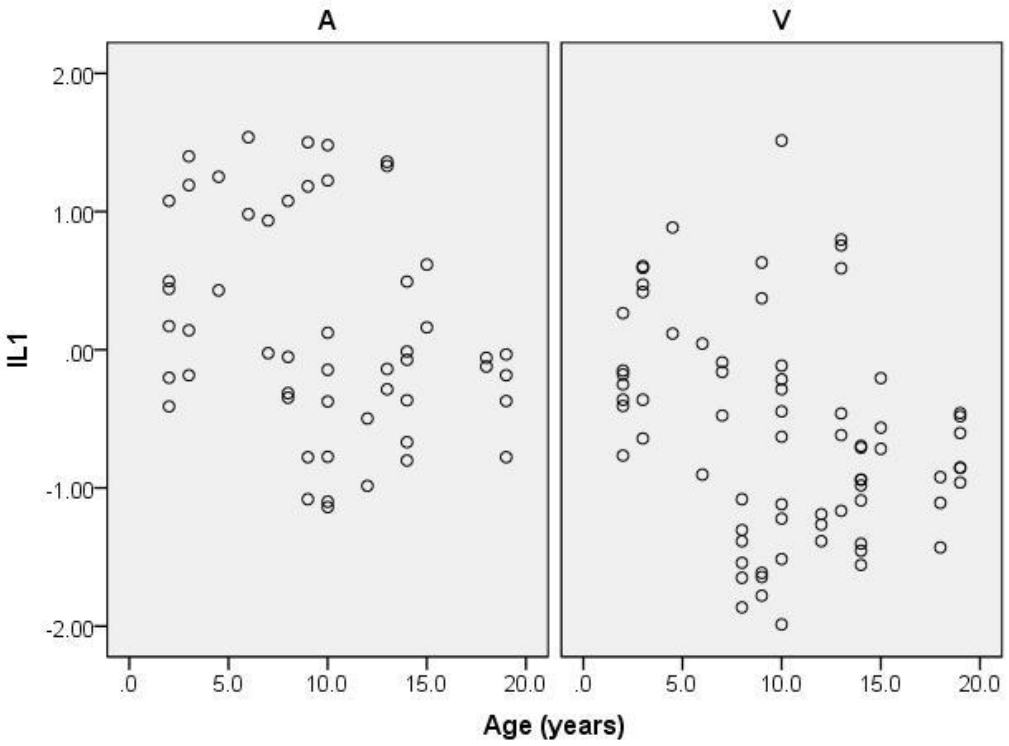
A
V
IL1
2.00
1.00
.00
-1.00
-2.00
.0
5.0
10.0
15.0
20.0
Age (years)

Fig. 2B

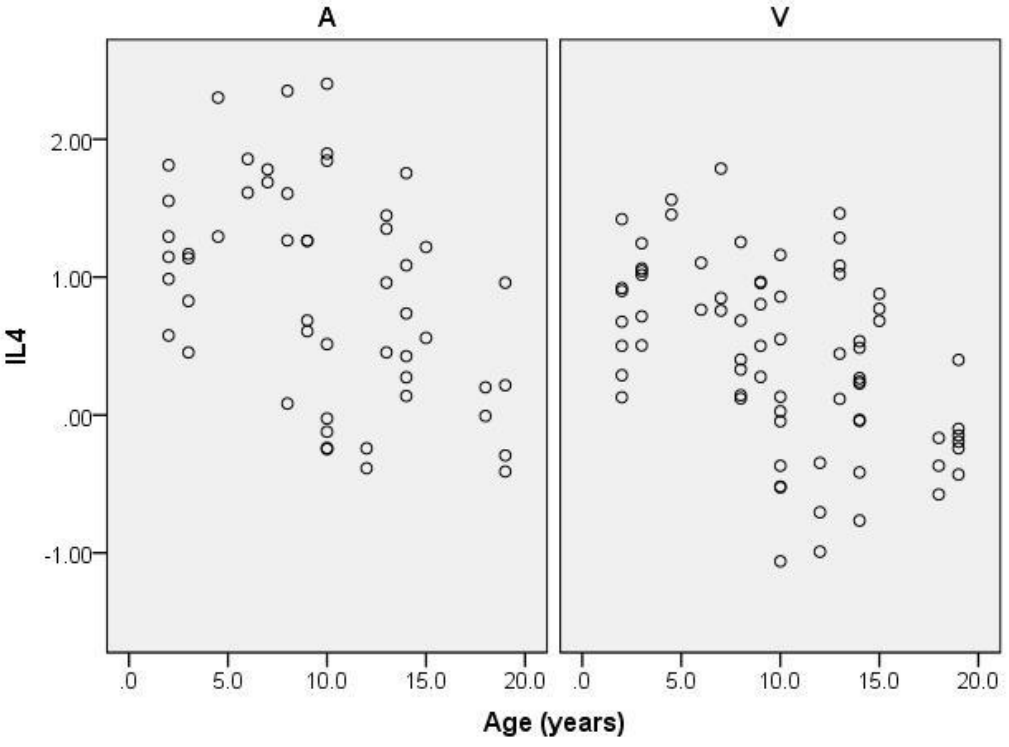
A
V
IL4
2.00
1.00
.00
-1.00
.0
5.0
10.0
15.0
20.0
Age (years)

Fig. 2C

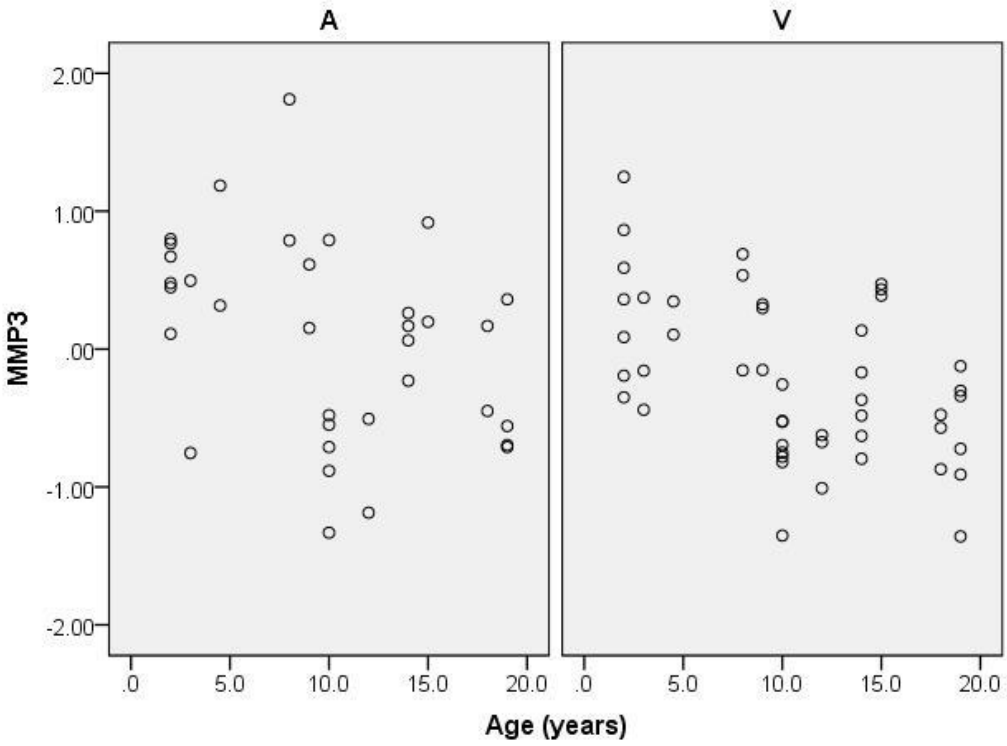
A
V
MMP3
2.00
1.00
.00
-1.00
-2.00
.0
5.0
10.0
15.0
20.0
Age (years)

Fig. 2D

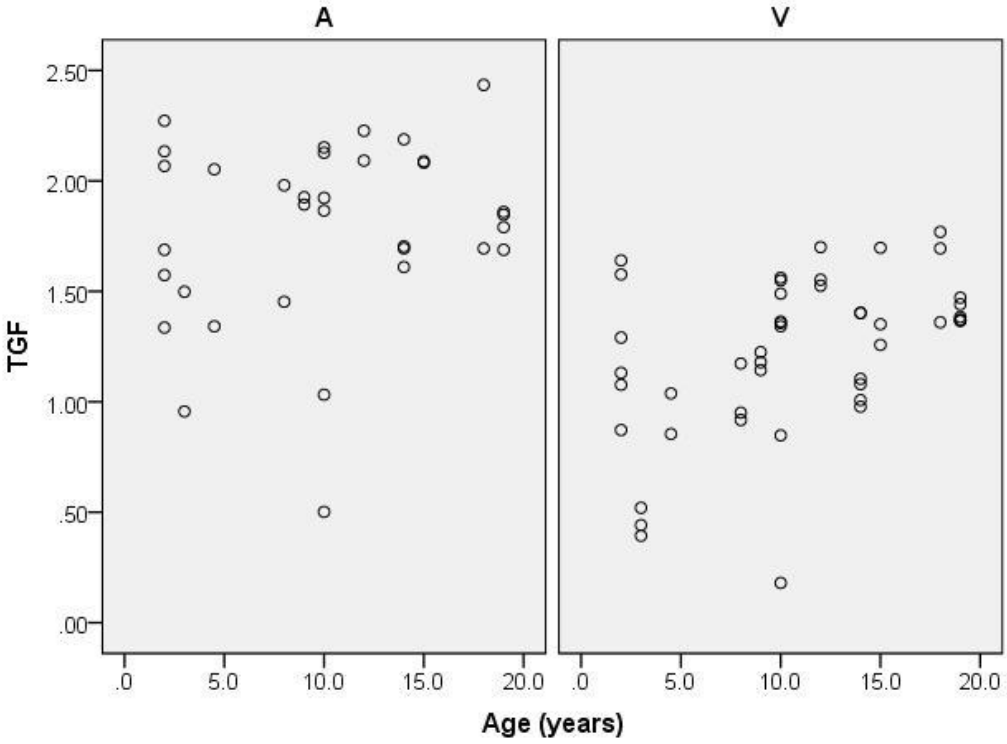
A
V
TGF
2.50
2.00
1.50
1.00
.50
.00
.0
5.0
10.0
15.0
20.0
Age (years)

Fig. 2 A-D. Scatterplots of age and relative IL-1 (**A**), IL-4 (**B**), MMP-3 (**C**) and TGF-β (**D**) mRNA expression in atria (A) and ventricles (V). A significant association of age and mRNA levels was present for atria and ventricular samples for **A.**IL-4 (p=0.01 and p=0.003, respectively) and for ventricular samples for **B.**IL-1 (p=0.019), **C.**MMP-3 (p=0.008) and **D.**TGF-β (p=0.001).

Comparing transcription levels of male and female cats showed significantly lower levels for IL-1 (p<0.001), IL-2 (p=0.002), IL-4 (p<0.001), IL-6 (p<0.001), IL-18 (p<0.001), TNF-α (p<0.001), MMP-3 (p=0.001), TIMP-1 (p<0.001) and TIMP-2 (p=0.006) in the entire myocardiumof female cats (Figure 3A, B). As for the entire cat population, both genders exhibitedgenerally higher transcription levels in the atria.

For female cats a negative association of age and myocardialIL-1 (p<0.001), IL-2 (p<0.001), IL-4 (p<0.001), IL-6 (p=0.001), IFN-γ (p=0.002), MMP-3 (p<0.001), and TIMP-2 (p=0.021) mRNA expression was observed, for male cats this applied for IL-6 (p=0.003), MMP-2 (p=0.034) and TIMP-1 (p=0.02).

Fig. 3A

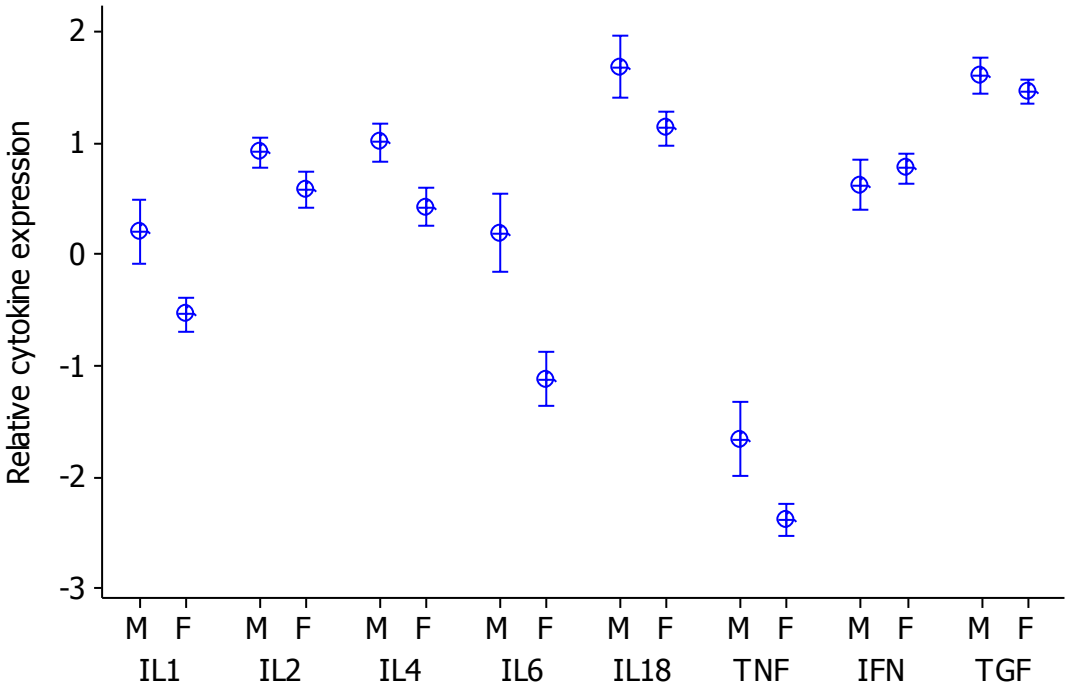

Fig. 3B

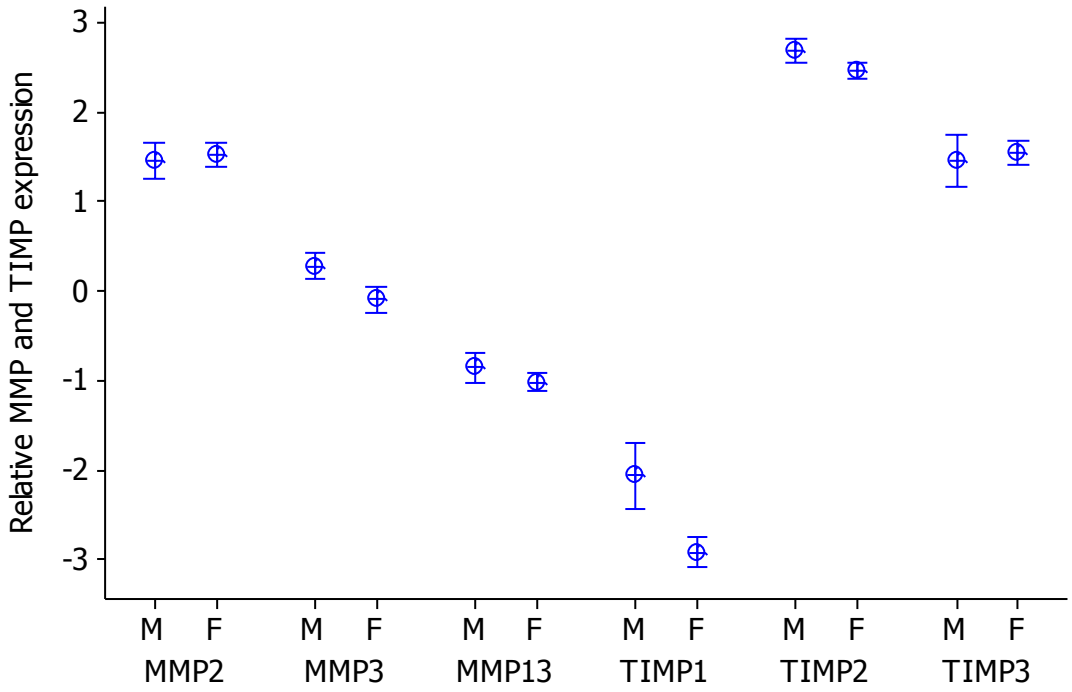

Fig. 3A, B. Relative myocardial mRNA expression of (**A**) IL-1, IL-2, IL-4, IL-6, TNF-α, IFN-γ, TGF-ß, and (**B**) MMP-2, MMP-3, MMP-13, TIMP-1, TIMP-2 and TIMP-3 comparing male (M) and female (F) cats. Significantly lower IL-1 ($p<0.001$), IL-2 ($p=0.002$), IL-4 ($p<0.001$), IL-6 ($p<0.001$), IL-18 ($p<0.001$), TNF-α ($p<0.001$), MMP-3 ($p=0.001$), TIMP-1 ($p<0.001$) and TIMP-2 ($p=0.006$) mRNA levels were present in the female myocardium. Error bars, bars represent 95% confidence interval for mean.

3.4. Non-cardiac systemic diseases are associated with increased myocardial cytokine, MMP and TIMP transcription

A comparison of the overall transcription levels of all markers in control cats and cats with systemic diseases showed significantly higher levels for IL-1 ($p=0.001$), IL-4 ($p=0.005$), IL-6 ($p=0.002$), IL-18 ($p<0.001$), TNF-α ($p=0.005$), MMP-13 ($p=0.006$), TIMP-1 ($p=0.04$), TIMP-2 ($p=0.019$) in cats with non-cardiac systemic diseases (Figure 4A, B). For IL-1, IL-6, IL-18 and TIMP-2 this applied to both atria and ventricles, whereas it applied to atria only for IL-4andMMP-13 and to ventricles only for TNF-α. For IL-2, IFN-γ, TGF-ß, MMP-2 and -3 as well as TIMP-1 and -3, differences were not significant.

Fig. 4A

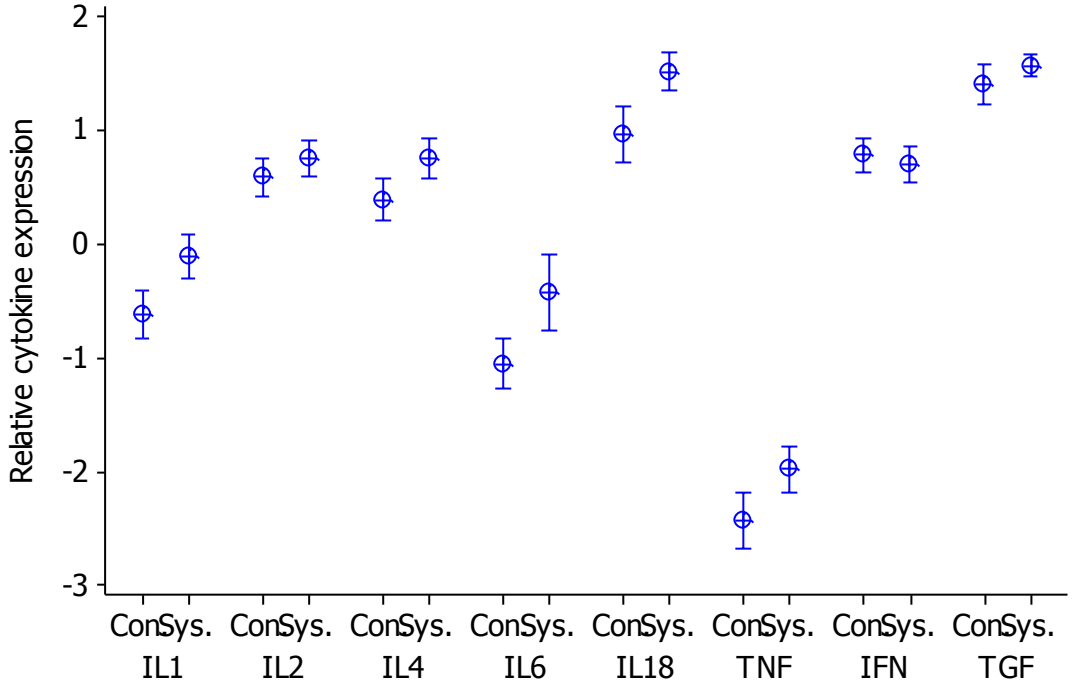
Relative cytokine expression
2
1
0
-1
-2
-3
Con
Sys.
IL1
IL2
IL4
IL6
IL18
TNF
IFN
TGF

Fig. 4B

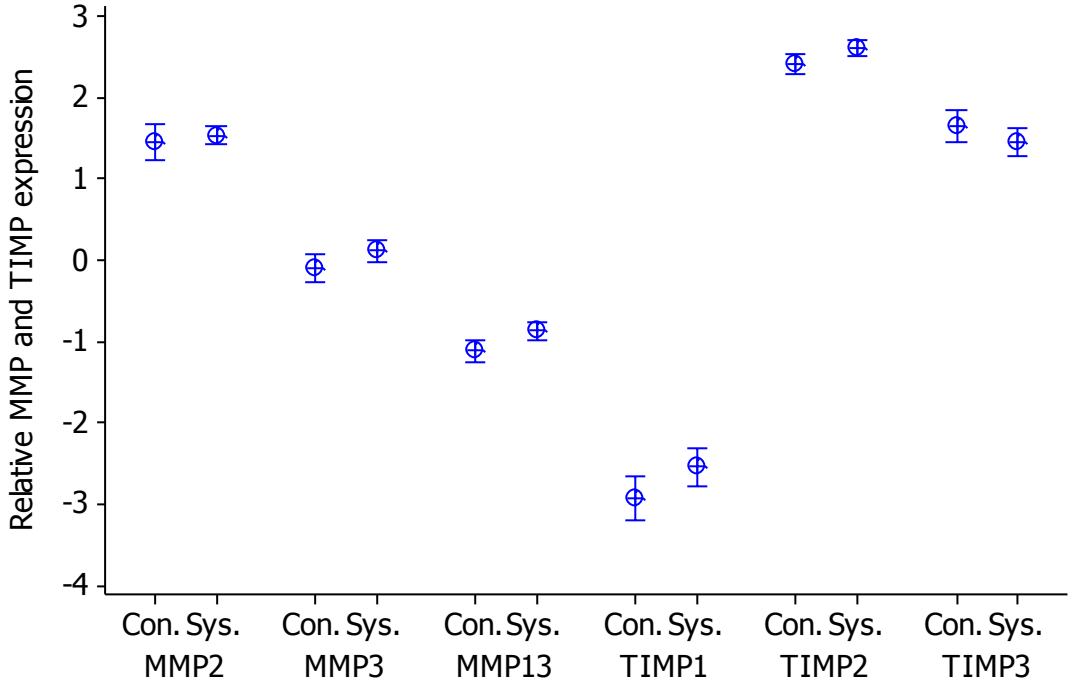
Relative MMP and TIMP expression
3
2
1
0
-1
-2
-3
-4
Con. Sys.
MMP2
MMP3
MMP13
TIMP1
TIMP2
TIMP3

Fig. 3A, B. Relative myocardial mRNA expression of (**A**) IL-1, IL-2, IL-4, IL-6, TNF-α, IFN-γ, TGF-ß, and (**B**) MMP-2, MMP-3, MMP-13, TIMP-1, TIMP-2 and TIMP-3 comparing "control cats" (Con.) and cats with non-cardiac "systemic diseases" (Sys.). Significantly higher mRNA levels for IL-1 (p=0.001), IL-4 (p=0.005), IL-6 (p=0.002), IL-18 (p<0.001), TNF-α (p=0.005), MMP-13 (p=0.006), TIMP-1 (p=0.04), TIMP-2 (p=0.019)were presentin cats with non-cardiac systemic diseases.Error bars, bars represent 95% confidence interval for mean.

4. Discussion

The present study assessed the transcription of cytokines and ECM remodelling enzymes in the feline heart and provides valuable basic information on myocardial function and reaction patterns. Our study shows that the myocardium of cats does indeed constitutively transcribe not only inflammatory and pro-fibrotic cytokines, i.e. IL-1, -2, -4, -6, -18, IFN-γ, TNF-α and TGF-β, but also ECM remodelling enzymes (MMP-2, -3, 13, TIMP-1, -2 and -3), with generally higher mRNA concentrations in atria than in ventricles. These results are in line not only with our previous findings in dogs (Fonfara et al., 2013a, b), where we could also confirm protein expression and identified the cardiomyocytes as the source of these markers (own observations), but also with a recent immunohistological study undertaken on cats (Aupperle et al., 2011). It suggests continuous remodelling of the feline myocardium at any age.

A younger age and male gender wereassociated with higher myocardial cytokine transcription. Furthermore, the age-associated myocardial reaction pattern differed between male and female cats.Considering that the majority of cats were neutered, other than hormonal factors seem to influence cardiac inflammation and remodelling (Chua et al., 2011; Pavon et al., 2012). In the absence of androgens as cause forcardiac inflammation, the higher myocardial levels of inflammatory and immune modulatory cytokines in the male cats might be associated with anappropriate myocardial response to the systemic diseases present in most cats, whereas the myocardium of female cats fails to develop a reactive response(Fang et al., 2007; Chen and Frangogiannis, 2010;Chua et al., 2011; Pavon et al., 2012). Similarly, a

younger age was associated with an increased transcription of inflammatory and immune modulatory cytokines. Several studies have confirmed the involvement of IL-1, IL-6, and IL-18 in myocardial inflammation, fibroblast proliferation, and increased collagen production(Colston et al., 2007; Chen et al., 2008; Yu et al., 2009; Hedayat et al., 2010; Tamariz and Hare, 2010; Fix et al., 2011). However, these cytokines are also involved in myocardial repair, compensatory ventricular hypertrophy and myocardial adjustment to changing haemodynamics (Colston et al., 2007; Yu et al., 2009; Hedayat et al., 2010; Fix et al., 2011).It is therefore possible that the observed inflammatory response is of benefit and enables myocardial repair (Chen and Frangogiannis, 2010; Chua et al., 2011). On the other hand, an increased inflammatory potential might predispose to myocardial damage and fibrosis, and cardiac diseases, i.e. hypertrophic cardiomyopathy, are more frequently diagnosed in male than female cats(Atkins et al., 1992; Abbott, 2010; Payne et al., 2010).

Whilst inflammatory cytokines reduced with age, TGF-β transcriptionincreasedsuggestinga profibrotic potential of the myocardium. In mouse models of ageing, increased TGF-β protein expression was seen in association with interstitial myocardial fibrosis and decreased ventricular compliance (Brooks and Conrad, 2000; Wang et al., 2010; Cai et al., 2012), andin cardiac diseases, TGF-β is known to be involved in the pathogenesis of cardiac remodelling and fibrosis (Khan and Sheppard, 2006; Dobaczewski et al., 2011).

Interestingly, age associated myocardial marker transcription differed between both sexes. Femalecats exhibited a reduction of IL-1, IL-2, IL-4, IL-6, IFN-γ, MMP-3 and TIMP-2 with age,in male cats this applied for IL-6, MMP-2 and TIMP-1.Conflicting results are reported for the role of immune modulatory cytokines in cardiac remodelling. In mouse models, Th2 induction resulted in reduced ventricular stiffness, and Th1 cells were involved in initiation of fibrosis and collagen crosslinking(Yu et al., 2005; 2010), whereasanother

study did detecta shift from Th1 to Th2 response in old mice in association with interstitial myocardial fibrosis (Cieslik et al., 2011). We observed a reduction of all three immune modulatory markers (IL-2, IL-4,IFN-γ)with age in thefemale cats, which might be consistent with a generally reduced myocardial inflammatory response, as suggested by thereduced transcriptionof IL-1 and IL-6.Aupperle and co-authors (2011) observed reduced TIMP-2 expression in association with myocardial fibrosis in cats.In older sheep (> 8 years), rat and mouse models, higher myocardial MMP-2 and reduced TIMP-2 mRNA and protein expression, as well as cardiomyocyte hypertrophy and perivascular and interstitial fibrosis, imbalanced cardiac remodelling and impaired repair was observed (Kandalam et al., 2010; Wang et al., 2010; Horn et al., 2012; Givvimani et al., 2013). The age-associated reduced TIMP-2 and MMP-2 transcription in female and male cats, respectively,suggests therefore a pro-fibrotic potential and impaired repair of the female myocardium, and further supports a reactive male myocardium facilitating appropriate repair (Davis et al., 2007; Chen and Frangogiannis, 2010).

Cats with diseases likely to have systemic effects showed overall higher myocardial transcription of IL-1, -6, -18 and TIMP-2, higher atrial transcription of IL-4 and MMP-13 and higher ventricular transcription for TNF-α than cats with diseases unlikely to have an effect on cardiac function. This suggests that systemic diseasescan induce and influence cardiac inflammation and remodelling, despite the lack of direct cardiac involvement in the disease and in the absence of cardiac histopathological changes. Similar observations have been reported for dogs (Fonfara et al., 2013a, b). Particular diseases, such as neoplasia, i.e. lymphoma, and inflammatory diseases have been shown to influence localised and circulating cytokine and MMP concentrations in dogs and cats (Van Nguyen et al., 2006; Janeczko et al., 2008; Stich and DeClue, 2011; Arico et al., 2013; Aresu et al., 2014). Although these studies did not investigate the heart, it is not unlikely that these also influence the myocardial

transcription. In our study, we could not detect any potential disease associated myocardial transcription pattern, which would suggest a more general, i.e. non-specific and pro-inflammatory effect on the heart as part of the general systemic effects of these non-cardiac diseases; however, further studies on larger animal cohorts would be required to thoroughly address this issue.

Interestingly, significant age-associated differences were predominantlyseen in the ventricles, whereas diseases with likely systemic effects seem to influence transcription levels in both atria and ventricles. Age-associated remodelling might therefore predominantly affect the ventricles, which might be involved in the development of heart failure with preserved ejection fraction (Santilli and Bussadori, 1998; Saunders, 2012; Loffredo et al., 2014). In contrast, diseases with systemic effects seem to influence the myocardium in general. The former might be the basis for regional differences in myocardial remodellingand the development of cardiac dysfunction and arrhythmia in patients with systemic diseases, as previously reported for dogs (Brundel, 2005; Fonfara et al., 2013a, b).

We are aware of the limitations of this study due to the small group sizes and the inhomogeneity of the groups, which all comprised diseased cats, though without any clinical and pathological evidence of cardiac disease. An influence on the detected transcription pattern in cats with disease conditions unlikely to affect the heart cannot be excluded completely. However, this is unlikely to be relevant, as differences in myocardial transcription between these cats and cats with diseases likely to have systemic effects were observed.We could not generate meaningful data on age-associated transcription patterns in control cats, since our control animal numbers were too low. Furthermore, a potential influence of diet and exercise was not considered in the cat population of our study.

5. Conclusion

The results of the present study suggest age-and gender-associated differences in the type and degree of cardiac remodelling. The myocardium of male and younger cats appears to be in a more pro-inflammatory basic state, whereas in female andolder cats a reduction in the inflammatory responsiveness is suggested. Furthermore, genderdifferences in age-associated cardiac transcription pattern were observed, despite the majority of cats being neutered. Further investigations are now needed to fully elucidate gender differences and the ageing processes of the feline myocardium, the myocardial reactivity to systemic non-cardiac diseases, and the potential association with cardiac diseases.

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References

Abbott, J.A., 2010. Feline hypertrophic cardiomyopathy: an update. Vet. Clin. North Am. Small Anim. Pract. 40, 685-700.

Aresu, L., Arico, A., Comazzi, S., Gelain, M.E., Riondato, F., Mortarino, M., Morello, E., Stefanello, D., Castagnaro, M., 2014. VEGF and MMP-9: biomarkers for canine lymphoma. Vet. Comp.Oncol. 12, 29-36.

Arico, A., Giantin, M., Gelain, M.E., Riondato, F., Comazzi, S., Rutgen, B.C., Essler, S.E., Dacasto, M., Castagnaro, M., Aresu, L., 2013. The role of vascular endothelial growth factor and matrix metalloproteinases in canine lymphoma: in vivo and in vitro study. BMC Vet. Res. 9, 94.

Ather, S., Chan, W., Bozkurt, B., Aguilar, D., Ramasubbu, K., Zachariah, A.A., Wehrens, X.H., Deswal, A., 2012. Impact of noncardiac comorbidities on morbidity and mortality in a predominantly male population with heart failure and preserved versus reduced ejection fraction. J Am. Coll. Cardiol. 59, 998-1005.

Atkins, C.E., Gallo, A.M., Kurzman, I.D., Cowen, P., 1992. Risk factors, clinical signs, and survival in cats with clinical diagnosis of idiopathic hypertrophic cardiomyopathy: 74 cases (1985-1989). J. Am. Vet. Med. Assoc. 201, 613-618.

Aupperle, H., Baldauf, K., Marz, I., 2011. An immunohistochemical study of feline myocardial fibrosis. JComp. Pathol. 145, 158-173.

Brooks, W.W., Conrad, C.H., 2000. Myocardial fibrosis in transforming growth factor beta(1)heterozygous mice. J Mol. Cell. Cardiol. 32, 187-195.

Brundel, B.J., Melynk, P., Rivard, L., Nattel, S., 2005. The pathology of atrial fibrillation in dogs. J Vet. Cardiol. 7, 121-129.

Cai, H., Yuan, Z., Fei, Q., Zhao, J., 2012. Investigation of thrombospondin-1 and transforming growth factor-beta expression in the heart of aging mice. Exp. Therap. Med. 3, 433-436.

Chen, D., Assad-Kottner, C., Orrego, C., Torre-Amione, G., 2008. Cytokines and acute heart failure. Crit. Care Med. 36, S9-16.

Chen, W., Frangogiannis, N.G., 2010. The role of inflammatory and fibrogenic pathways in heart failure associated with aging. Heart Fail.Rev. 15, 415-422.

Chua, S., Wang, H.L., Lin, Y.C., Wu, C.H., Tsai, T.H., Chang, L.T., Kao, Y.H., Yen, C.H., Yip, H.K., Sun, C.K., 2011. Enhanced Expression of Plasminogen Activator Inhibitor May Prevent Cardiac Rupture in Female and Castrated Mice After Myocardial Infarction. Gender Med. 8, 239-251.

Cieslik, K.A., Taffet, G.E., Carlson, S., Hermosillo, J., Trial, J., Entman, M.L., 2011. Immune-inflammatory dysregulation modulates the incidence of progressive fibrosis and diastolic stiffness in the aging heart. J Mol. Cell. Cardiol. 50, 248-256.

Colston, J.T., Boylston, W.H., Feldman, M.D., Jenkinson, C.P., de la Rosa, S.D., Barton, A., Trevino, R.J., Freeman, G.L., Chandrasekar, B., 2007. Interleukin-18 knockout mice display maladaptive cardiac hypertrophy in response to pressure overload. Biochem.Biophys. Res.Commun. 354, 552-558.

Davis, D.R., Wilson, K., Sam, M.J., Kennedy, S.E., Mackman, N., Charlesworth, J.A., Erlich, J.H., 2007. The development of cardiac fibrosis in low tissue factor mice is gender-dependent and is associated with differential regulation of urokinase plasminogen activator. J. Mol.Cell.Cardiol. 42, 559-571.

Dobaczewski, M., Chen, W., Frangogiannis, N.G., 2011. Transforming growth factor (TGF)-beta signaling in cardiac remodeling. J Mol. Cell.Cardiol. 51, 600-606.

Fang, L., Gao, X.M., Moore, X.L., Kiriazis, H., Su, Y., Ming, Z., Lim, Y.L., Dart, A.M., Du, X.J., 2007. Differences in inflammation, MMP activation and collagen damage account for gender difference in murine cardiac rupture following myocardial infarction. J. Mol.Cell.Cardiol.43, 535-544.

Fix, C., Bingham, K., Carver, W., 2011. Effects of interleukin-18 on cardiac fibroblast function and gene expression. Cytokine 53, 19-28.

Fonfara, S., Hetzel, U., Tew, S.R., Cripps, P., Dukes-McEwan, J., Clegg, P.D., 2013a. Myocardial cytokine expression in dogs with systemic and naturally occurring cardiac diseases. Am. J Vet.Res. 74, 408-416.

Fonfara, S., Hetzel, U., Tew, S.R., Cripps, P., Dukes-McEwan, J., Clegg, P.D., 2013b. Expression of matrix metalloproteinases, their inhibitors, and lysyl oxidase in myocardial samples from dogs with end-stage systemic and cardiac diseases. Am. J Vet.Res. 74, 216-223.

Fonfara, S., Tew, S.R., Cripps, P., Dukes-McEwan, J., Clegg, P.D., 2012. Increased blood mRNA expression of inflammatory and anti-fibrotic markers in dogs with congestive heart failure. Res. Vet.Sci. 93, 879-885.

Givvimani, S., Kundu, S., Narayanan, N., Armaghan, F., Qipshidze, N., Pushpakumar, S., Vacek, T.P., Tyagi, S.C., 2013. TIMP-2 mutant decreases MMP-2 activity and augments pressure overload induced LV dysfunction and heart failure. Arch. Physiol. Biochem. 119, 65-74.

Hedayat, M., Mahmoudi, M.J., Rose, N.R., Rezaei, N., 2010. Proinflammatory cytokines in heart failure: double-edged swords. Heart Fail. Rev. 15, 543-562.

Horn, M.A., Graham, H.K., Richards, M.A., Clarke, J.D., Greensmith, D.J., Briston, S.J., Hall, M.C., Dibb, K.M., Trafford, A.W., 2012. Age-related divergent remodeling of the cardiac extracellular matrix in heart failure: collagen accumulation in the young and loss in the aged. J Mol. Cell. Cardiol. 53, 82-90.

Janeczko, S., Atwater, D., Bogel, E., Greiter-Wilke, A., Gerold, A., Baumgart, M., Bender, H., McDonough, P.L., McDonough, S.P., Goldstein, R.E., Simpson, K.W., 2008. The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. Vet. Microbiol. 128, 178-193.

Kandalam, V., Basu, R., Abraham, T., Wang, X., Soloway, P.D., Jaworski, D.M., Oudit, G.Y., Kassiri, Z., 2010. TIMP2 deficiency accelerates adverse post-myocardial infarction remodeling because of enhanced MT1-MMP activity despite lack of MMP2 activation. Circ. Res. 106, 796-808.

Khan, R., Sheppard, R., 2006. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. Immunol. 118, 10-24.

Kipar, A., Leutenegger, C.M., Hetzel, U., Akens, M.K., Mislin, C.N., Reinacher, M., Lutz, H., 2001. Cytokine mRNA levels in isolated feline monocytes. Vet. Immunol. Immunopathol. 78, 305-315.

Kitzman, D.W., Edwards, W.D., 1990. Age-related changes in the anatomy of the normal human heart. J. Gerontol. 45, M33-39.

Lindsey, M.L., Goshorn, D.K., Squires, C.E., Escobar, G.P., Hendrick, J.W., Mingoia, J.T., Sweterlitsch, S.E., Spinale, F.G., 2005. Age-dependent changes in myocardial matrix metalloproteinase/tissue inhibitor of metalloproteinase profiles and fibroblast function. Cardiovasc. Res. 66, 410-419.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Loffredo, F.S., Nikolova, A.P., Pancoast, J.R., Lee, R.T., 2014. Heart failure with preserved ejection fraction: molecular pathways of the aging myocardium. Circ. Res. 115, 97-107.

Maggio, M., Guralnik, J.M., Longo, D.L., Ferrucci, L., 2006. Interleukin-6 in aging and chronic disease: a magnificent pathway. JGerontol. A Biol. Sci. Med. Sci. 61, 575-584.

Metzger, F.L., Rebar, A.H., 2012. Clinical pathology interpretation in geriatric veterinary patients. Vet. Clin. North Am. Small Anim. Pract.42, 615-629, v.

Morley, J.E., Baumgartner, R.N., 2004. Cytokine-related aging process. JGerontol. A Biol. Sci. Med. Sci. 59, M924-929.

Pavon, N., Martinez-Abundis, E., Hernandez, L., Gallardo-Perez, J.C., Alvarez-Delgado, C., Cerbon, M., Perez-Torres, I., Aranda, A., Chavez, E., 2012. Sexual hormones: effects on cardiac and mitochondrial activity after ischemia-reperfusion in adult rats. Gender difference. J.Steroid Biochem. Mol. Biol. 132, 135-146.

Payne, J., Luis Fuentes, V., Boswood, A., Connolly, D., Koffas, H., Brodbelt, D., 2010. Population characteristics and survival in 127 referred cats with hypertrophic cardiomyopathy (1997 to 2005). J. Small Anim. Pract. 51, 540-547

Santilli, R.A., Bussadori, C., 1998. Doppler echocardiographic study of left ventricular diastole in non-anaesthetized healthy cats. Vet. J 156, 203-215.

Saunders, A.B., 2012. The diagnosis and management of age-related veterinary cardiovascular disease. Vet. Clin. North Am. Small Anim. Pract.42, 655-668, vi.

Schober, K.E., Fuentes, V.L., 2001. Effects of age, body weight, and heart rate on transmitral and pulmonary venous flow in clinically normal dogs. Am. J Vet.Res.62, 1447-1454.

Spinale, F.G., 2007. Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. Physiol. Rev. 87, 1285-1342.

Stich, A.N., DeClue, A.E., 2011. Pathogen associated molecular pattern-induced TNF, IL-1beta, IL-6 and CXCL-8 production from feline whole blood culture. Res. Vet. Sci. 90, 59-63.

Taglinger, K., Van Nguyen, N., Helps, C.R., Day, M.J., Foster, A.P., 2008. Quantitative real-time RT-PCR measurement of cytokine mRNA expression in the skin of normal cats and cats with allergic skin disease. Vet. Immunol. Immunopathol. 122, 216-230.

Tamariz, L., Hare, J.M., 2010. Inflammatory cytokines in heart failure: roles in aetiology and utility as biomarkers. Europ. Heart J 31, 768-770.

Tian, H., Cimini, M., Fedak, P.W., Altamentova, S., Fazel, S., Huang, M.L., Weisel, R.D., Li, R.K., 2007. TIMP-3 deficiency accelerates cardiac remodeling after myocardial infarction. J Mol. Cell. Cardiol. 43, 733-743.

Uchinaka, A., Kawaguchi, N., Mori, S., Hamada, Y., Miyagawa, S., Saito, A., Sawa, Y., Matsuura, N., 2014. Tissue Inhibitor of Metalloproteinase-1 and -3 Improves Cardiac Function in an Ischemic Cardiomyopathy Model Rat. Tissue Eng. Part A. 20, 3073-84.

Van Nguyen, N., Taglinger, K., Helps, C.R., Tasker, S., Gruffydd-Jones, T.J., Day, M.J., 2006. Measurement of cytokine mRNA expression in intestinal biopsies of cats with inflammatory enteropathy using quantitative real-time RT-PCR. Vet. Immunol. Immunopathol. 113, 404-414.

Vanhoutte, D., Heymans, S., 2010. TIMPs and cardiac remodeling: 'Embracing the MMP-independent-side of the family'. J Mol. Cell. Cardiol.48, 445-453.

Walston, J.D., Matteini, A.M., Nievergelt, C., Lange, L.A., Fallin, D.M., Barzilai, N., Ziv, E., Pawlikowska, L., Kwok, P., Cummings, S.R., Kooperberg, C., LaCroix, A., Tracy, R.P., Atzmon, G., Lange, E.M., Reiner, A.P., 2009. Inflammation and stress-related candidate genes, plasma interleukin-6 levels, and longevity in older adults. Exp. Gerontol. 44, 350-355.

Wang, M., Zhang, J., Walker, S.J., Dworakowski, R., Lakatta, E.G., Shah, A.M., 2010. Involvement of NADPH oxidase in age-associated cardiac remodeling. J Mol. Cell. Cardiol.48, 765-772.

Yu, Q., Vazquez, R., Khojeini, E.V., Patel, C., Venkataramani, R., Larson, D.F., 2009. IL-18 induction of osteopontin mediates cardiac fibrosis and diastolic dysfunction in mice. Am. J Physiol. Heart Circ. Physiol. 297, H76-85.

Yu, Q., Vazquez, R., Zabadi, S., Watson, R.R., Larson, D.F., 2010. T-lymphocytes mediate left ventricular fibrillar collagen cross-linking and diastolic dysfunction in mice. Matrix Biol. 29, 511-518.

Yu, Q., Watson, R.R., Marchalonis, J.J., Larson, D.F., 2005. A role for T lymphocytes in mediating cardiac diastolic function. Am. J Physiol. Heart Circ. Physiol. 289, H643-651.